

ORIGINAL ARTICLE

MiR-152 suppresses the proliferation and invasion of NSCLC cells by inhibiting FGF2

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MicroRNAs (miRNAs) regulate the proliferation and metastasis of cancer cells. Here, we showed that miR-152 was downregulated in non-small-cell lung cancer (NSCLC) tissues and cell lines. Overexpression of miR-152 suppressed cell proliferation and colony formation and also limited migration and invasion. Fibroblast growth factor 2 (FGF2) was confirmed as a direct target of miR-152. FGF2 knockdown suppressed cell proliferation, colony formation, migration and invasion, whereas FGF2 overexpression partially reversed the suppressive effect of miR-152. Furthermore, the presence of miR-152 was inversely correlated with FGF2 in NSCLC tissues. Overall, this study demonstrated that miR-152 suppressed the proliferation and invasion of NSCLC cells by downregulating FGF2. These findings provide novel insights with potential therapeutic applications for the treatment of NSCLC.

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INTRODUCTION

Lung cancer is the most common cause of cancer-related death worldwide. Its incidence is rapidly increasing in developing countries, with non-small-cell lung cancer (NSCLC) accounting for >80% of all lung cancer cases.¹ The prognosis for NSCLC remains poor despite recent advances in the diagnosis of and chemotherapies used for this cancer, and the 5-year overall survival rate of NSCLC is a dismal 11%.² Thus, the elucidation of the molecular mechanisms that control NSCLC tumor metastasis is urgently needed.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that negatively regulate the expression of their target genes by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs that leads to mRNA degradation or translational suppression.^{3,4} The miRNAs regulate the expression of multiple target genes involved in various biological processes, including cell proliferation, differentiation, migration and apoptosis.^{5,6} Recently, mounting evidence has indicated that aberrant changes in miRNA expression correlates with a wide range of cancers and that miRNAs act as oncogenes and tumor suppressors.^{7,8} In NSCLC, multiple miRNAs, such as miR-10b, miR-150 and miR-205, were found to promote NSCLC carcinogenesis.^{9–11} In contrast, miR-16, miR-140 and miR-223 have been identified as tumor suppressors.^{7,12,13} In several cancers, including NSCLC,¹⁴ miR-152 levels are decreased, whereas miR-152 functions as a tumor suppressor in cancers

including prostate cancer, glioma and endometrial cancer.^{15–17} Recently, Su *et al.*¹⁸ showed that miR-152 suppresses NSCLC progression by targeting ADAM metalloproteinase domain 17 (ADAM17). However, the function of miR-152 in NSCLC remains poorly understood.

In the present study, we found that miR-152 was significantly decreased in NSCLC, whereas miR-152 overexpression significantly suppressed the proliferation and motility of NSCLC cells. Furthermore, we found that miR-152 directly targeted fibroblast growth factor 2 (FGF2) in NSCLC cells and that miR-152 was inversely correlated with FGF2 in NSCLC tissues. Together, these results suggest that miR-152 could be a therapeutic and prognostic target for the treatment of NSCLC.

MATERIALS AND METHODS

Patient samples and cell lines

Human NSCLCs and matched normal tissues were obtained from 30 patients in our department. Informed written consent was obtained from all patients, and this study was approved by the Medical Ethics Committee at Zhongnan Hospital. All tissue samples were stored in liquid nitrogen until RNA extraction and western blotting. Clinicopathologic information is summarized in Table 1. The NSCLC cell lines A549, SK-MES-1, H460 and H520 and the normal lung bronchus epithelial cell line 16HBE were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cells

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Table 1 Relationship between microRNA-152 (miR-152) and clinicopathological variables in lung cancer tissues

Variable	N	Low	High	P-value
Age (years)				0.135 ^a
≥60	18	10	8	
<60	12	5	7	
Gender				0.414 ^a
Male	16	9	7	
Female	14	6	8	
Size				0.326 ^a
>3 cm	19	9	10	
≤3 cm	11	6	5	
Histology type				0.341 ^a
Adenocarcinoma	17	8	9	
Squamous cancer	13	7	6	
Histological grade				0.286 ^b
I	13	6	7	
II	8	3	5	
III	9	5	4	0.024 ^b
Pathological stage				
I	12	4	8	
II	7	3	4	
III	11	8	3	
Lymph node status				0.014 ^a
Metastasis	14	9	5	
No metastasis	16	6	10	

^aThe χ^2 test.^bMann-Whitney test.

were maintained in a humidified, 5% CO₂ atmosphere at 37 °C. After the NSCLC cells reached 80% confluence, they were transfected with miR-152 or control mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Plasmids and luciferase activity assays

MiR-152 and control mimics/inhibitors were purchased from Ribio Bio (Guangzhou, China). FGF2 short hairpin RNA (shRNA) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For FGF2 overexpression, the coding sequence of FGF2 was PCR-amplified and cloned into pcDNA4.0. For measuring the effect of miR-152 on the 3'-UTR of FGF2, we generated a luciferase expression construct containing part of FGF2 3'-UTR. We amplified the wild-type fragment 2132-3768 of FGF2 mRNA that contained potential miR-152-binding sites at position 3099-3121, using the following primers: 5'-GGGGTACCACTGAGCCATAATCGTGC-3' (forward) and 5'-CCCTCGAGAACAACCTCACCTACCCAGA-3' (reverse). The PCR fragment was inserted into the pGL3 Basic Vector using the *KpnI/XhoI* endonuclease restriction sites. Mutation of the FGF 3'-UTR (Mut) was performed using a mutation kit (Stratagene, La Jolla, CA, USA).

For luciferase activity assays, 50% confluent HEK293 cells were co-transfected with 100 ng of wild-type or Mut FGF2 3'-UTR and 100 nM miR-152 or control mimics using Lipofectamine 2000.

Cells were also transformed with 100 ng of PGL3 control vector to monitor transfection efficiency. MiR-NC was used as a nontargeting negative control. In addition, all cells were transfected with pRL-TK (Promega, Madison, WI, USA) as a control for normalization. Cells were harvested 48 h after transfection for luciferase activity assays using the dual-luciferase reporter assay system (Promega). Relative firefly luciferase activity was obtained by normalization to *Renilla* luciferase activity.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. FGF2 expression was detected with SYBR Green reagents (TAKARA, Tokyo, Japan) using the following primers: 5'-AGGAGAGCGACCCACACATCAA-3' (forward) and 5'-AGCCAG-CAGTCTTCCATCTTCC-3' (reverse). MiRNA was extracted using an All-in-One microRNA extraction kit and detected with an All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Carlsbad, CA, USA) using SYBR Green reagents. Primers for miR-152 (Cat No. HmiRQP3058) and U6 (HmiRQP9001) were purchased from GeneCopoeia. FGF2 expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and miR-152 was normalized to U6. Expression levels were quantified using the 2^{- $\Delta\Delta C_t$} method.

Cell survival assays

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). Briefly, 5 × 10³ cells were cultured in 96-well plates. Then, 24 h after transfection, 10 μ l of CCK-8 reagent was added to each well and incubated at 37 °C for 1.5 h. Absorbance at 450 nm was detected using a microtiter plate reader.

Colony formation assay

To assess colony formation, 24 h after transfection, 500 cells were plated in 6-well plates and grown for 2 weeks; the culture medium was replaced every 4 days. Cells were fixed with methanol and stained with 0.5% crystal violet for 20 min; visible colonies were counted. Triplicate wells were measured for each group.

Flow cytometry

Cell apoptosis was assayed using flow cytometry. Briefly, treated cells were trypsinized, collected, washed and stained with Annexin V-fluorescein isothiocyanate and propidium iodide for 15 min at 4 °C. The stained cells were analyzed with a flow cytometer (FACScalibur, BD, Franklin Lakes, NJ, USA).

Migration and invasion

Migration and invasion assays were performed using Transwell chambers with a pore size of 8 μ m. Cells were transfected with miR-152 or control mimics and incubated for 24 h. For migration assays, 5 × 10⁴ transfected cells were placed in the upper chamber. Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added to the lower chamber as a chemoattractant. Chambers were incubated at 37 °C in 5% CO₂ for 16 h, and then cells on the upper surface were removed. Cells that had migrated to the bottom surface were washed twice with cold phosphate-buffered saline, fixed in methanol and stained with 0.1% crystal violet. Stained cells were counted under a microscope. For invasion assays, the upper chamber was precoated with Matrigel. Both assays were performed in three independent experiments.

Western blotting

Cultured cells were washed three times in cold phosphate-buffered saline and lysed with RIPA buffer (pH 7.4, 50 mM Tris-HCl, 1% (v/v) Triton X-100, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM Na₃VO₄ and 10 mM NaF). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with primary antibodies overnight at 4 °C followed by secondary antibodies for 1 h at room temperature. Blots were visualized using an ECL detection system (Amersham, Piscataway, NJ, USA) and analyzed by Kodak Digital Science 1D software (Eastman Kodak, Rochester, NY, USA). Image J software (National institutes of Health, Bethesda, MD, USA) was used to quantify the intensity of protein bands. The relative intensity was calculated by normalization to GAPDH that was used as a loading control. Experiments were performed in triplicate.

Statistical analysis

Data are presented as the mean ± s.d. Student's *t*-test or analysis of variance was performed for statistical analyses using SPSS 12.0 (Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

RESULTS

The miR-152 levels were decreased in NSCLC tissues and cell lines

The expression of miR-152 in 30 pairs of NSCLC tissues and their matched normal tissues was measured using qRT-PCR. The results showed that miR-152 expression was significantly downregulated in NSCLC tissues compared with matched controls (Figure 1a). In addition, the expression of miR-152 in four NSCLC cell lines was determined. We found that the relative expression of miR-152 in these NSCLC cells was strikingly decreased compared with that of the normal lung bronchus epithelial cell line 16HBE (Figure 1b). These results suggest that downregulation of miR-152 may contribute to the progression of NSCLC.

MiR-152 suppressed proliferation of NSCLC cells

To understand the role of miR-152 in the development of NSCLC, miR-152 was transfected into A549 cells, and we then used a CCK-8 assay to determine their proliferation. We found

that miR-152 overexpression significantly suppressed A549 cell proliferation compared with their corresponding controls (Figure 2a). Next, flow cytometry was used to examine the apoptosis of A549 cells, and we found that miR-152 overexpression also induced this process (Figure 2b). We also used a colony formation assay to test the possibility that alterations in gene expression could cause either cell cycle arrest or cell death and lead to a reduction in colony number. Indeed, exogenous miR-152 expression significantly inhibited A549 colony formation (Figure 2c). The expression of miR-152 after transfection with a miR-152 mimic was determined by qRT-PCR (Figure 2d). Taken together, these results suggest that miR-152 was able to suppress NSCLC cell growth and induce apoptosis.

MiR-152 suppressed NSCLC cell migration and invasion

Migration and invasion are fundamental functions underlying several cellular processes, including angiogenesis, immune response and metastasis of cancer cells. Here, Transwell chambers were used to measure the number of cells that traversed a porous membrane or moved through an extra-cellular matrix. These assays revealed that overexpression of miR-152 significantly decreased migration (Figure 3a) and invasion (Figure 3b) of A549 cells, suggesting that miR-152 suppressed the motility of NSCLC cells.

FGF2 was a direct target of miR-152

To identify targets of miR-152, we used TargetScan 6.2 (<http://www.targetscan.org>), a widely used miRNA target prediction website. FGF2 was found to be a potential target (Figure 4a). Luciferase activity assays are commonly used to validate the suppressive effects of miRNAs on their target mRNAs. Here, we found that miR-152 significantly inhibited the luciferase activity of the wild-type but not the Mut 3'-UTR of FGF2 in HEK293 cells (Figure 4b). Moreover, overexpression of miR-152 significantly suppressed levels of both FGF2 mRNA and protein, whereas the inhibition of miR-152 significantly increased these levels (Figures 4c and d). These data suggest

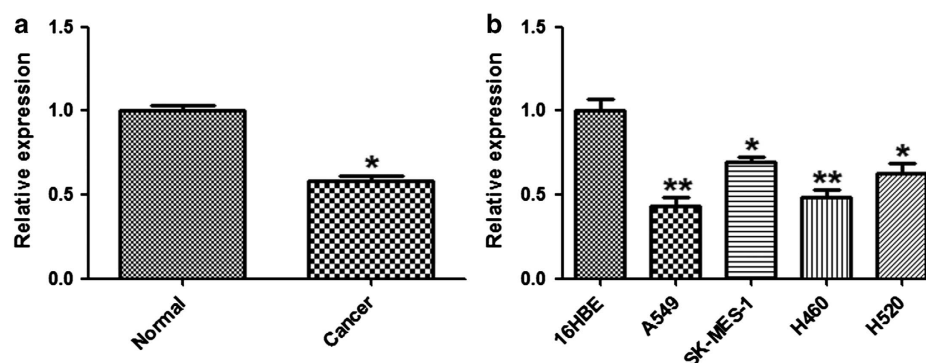


Figure 1 MicroRNA-152 (miR-152) was decreased in non-small-cell lung cancer (NSCLC) tissues and cell lines. (a) The expression levels of miR-152 in 30 pairs of NSCLC tissues and their matched normal tissues were measured by quantitative real-time PCR (qRT-PCR). U6 was used as an internal control. (b) The expression levels of miR-152 in a normal lung epithelial cell line (16HBE) and four NSCLC cell lines (A549, SK-MES-1, H460 and H520). **P* < 0.05, ***P* < 0.01 vs control.

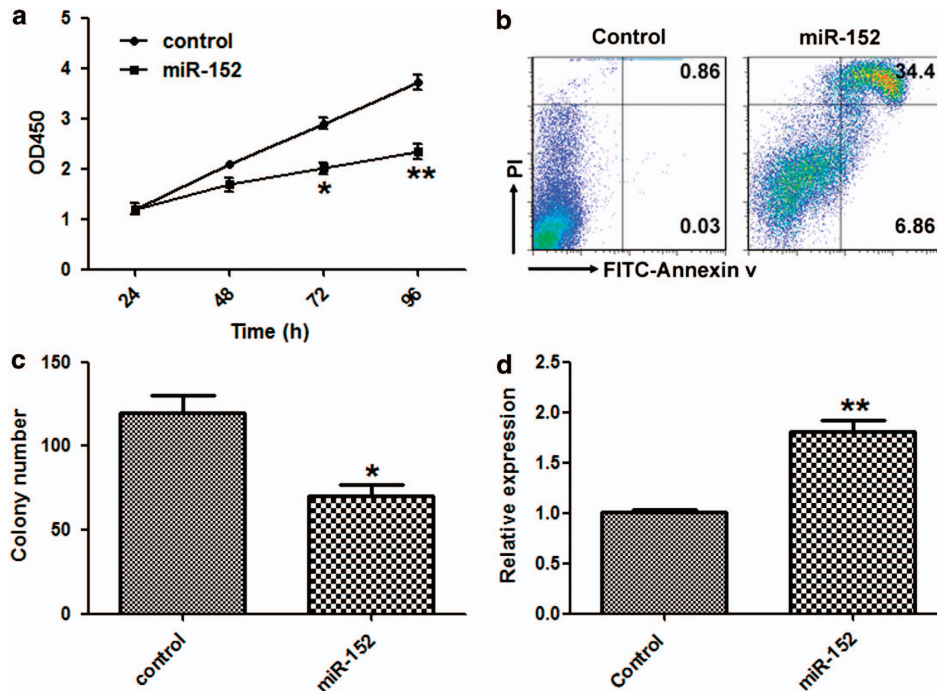


Figure 2 MicroRNA-152 (miR-152) suppressed the proliferation of non-small-cell lung cancer (NSCLC) cells. (a) A549 cells were transfected with miR-152 or control mimics, and a cell viability assay (CCK-8) was performed. (b) Cell apoptosis assays. (c) Colony formation. (d) Expression of miR-152 in A549 cells transfected with miR-152 or control mimics. * $P < 0.05$, ** $P < 0.01$ vs control.

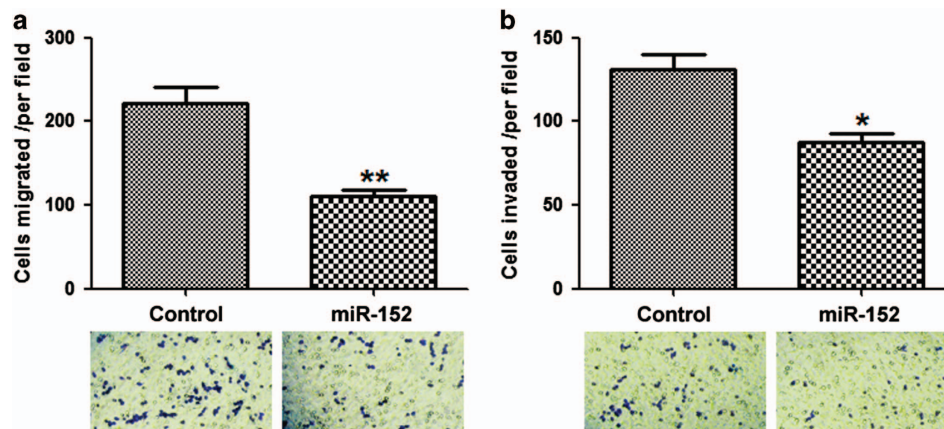


Figure 3 MicroRNA-152 (miR-152) suppressed the migration and invasion of non-small-cell lung cancer (NSCLC) cells. (a) A549 cells were transfected with miR-152 or control mimics, and *in vitro* migration was assessed. (b) *In vitro* invasion assay. * $P < 0.05$, ** $P < 0.01$ vs control.

that miR-152 might induce the degradation of FGF2 mRNA, leading to a reduction in levels of FGF2 protein and implying that FGF2 is a direct target of miR-152.

FGF2 inhibition resulted in similar effects as miR-152 overexpression

Because we found that FGF2 is a direct target of miR-152, we further investigated whether FGF2 silencing by shRNA could induce effects on NSCLC cells similar to those caused by the overexpression of miR-152. A549 cells were transfected with either an shRNA targeted against FGF2 or a nontargeting

control. The inhibition of FGF2 by shRNA significantly suppressed the proliferation, migration and invasion of A549 cells (Figures 5a–c). Western blotting and qRT-PCR were used to detect the effects of FGF2 shRNA (Figures 5d and e). These data indicate that inhibition of FGF2 mimicked the effects of miR-152 overexpression.

FGF2 overexpression partially attenuated the tumor suppressive effect of miR-152

We further investigated whether FGF2 overexpression could attenuate the tumor suppressive effects of miR-152. Indeed,

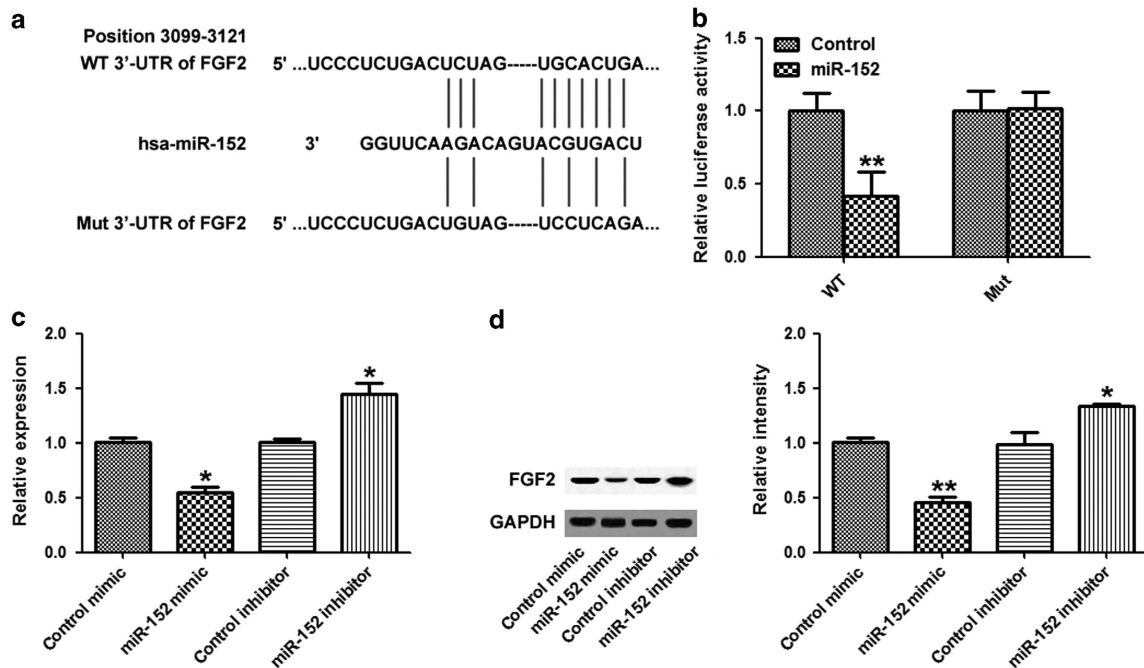


Figure 4 Fibroblast growth factor 2 (FGF2) was a direct target of microRNA-152 (miR-152). (a) Schematic representation of wild-type (WT) and mutated (Mut) putative miR-152-binding sites in the 3'-untranslated region (3'-UTR) of FGF2. (b) HEK293 cells were co-transfected with 100 nM WT or Mut FGF2 3'-UTR, pGL-3 control and either miR-152 or negative control mimics. Relative firefly luciferase activity was normalized to *Renilla* luciferase activity. (c) Expression of FGF2 mRNA was detected by quantitative real-time PCR (qRT-PCR) in A549 cells transfected with miR-152 or control mimic and in A549 cells transfected with miR-152 or control inhibitor. (d) Protein levels were detected by western blot analysis in A549 cells transfected with miR-152 or control mimic and in A549 cells transfected with miR-152 or control inhibitor. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. * $P < 0.05$, ** $P < 0.01$ vs control.

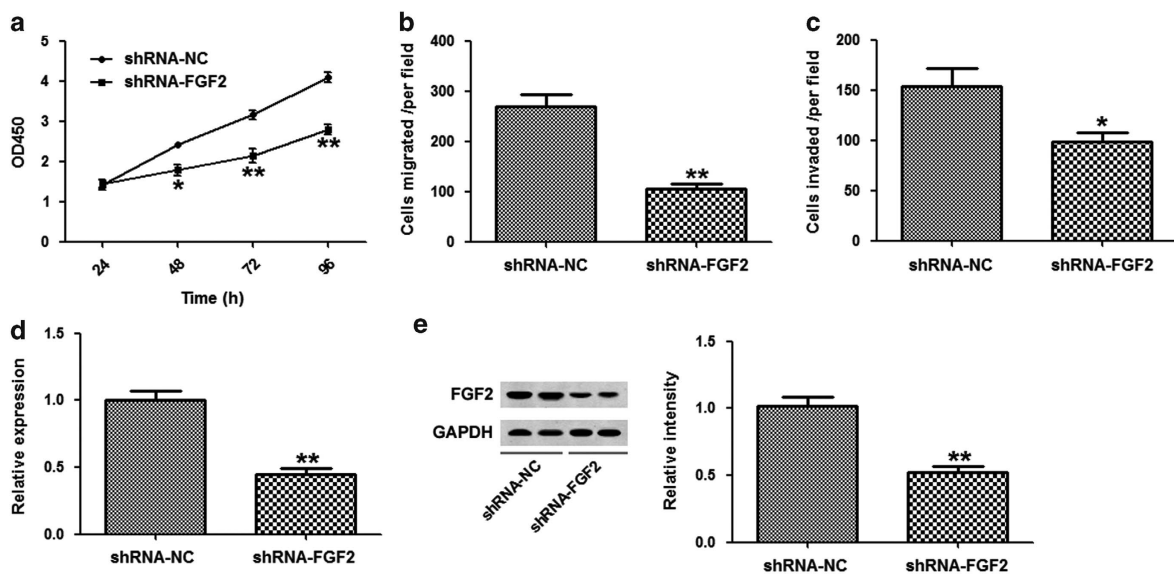


Figure 5 Fibroblast growth factor 2 (FGF2) inhibition resulted in similar effects as microRNA-152 (miR-152) overexpression. (a) The viability of A549 cells transfected with FGF2 or control short hairpin RNAs (shRNAs) was detected using CCK-8. (b) *In vitro* migration. (c) *In vitro* invasion. (d) Expression of FGF2 mRNA was detected by quantitative real-time PCR (qRT-PCR) in A549 cells transfected with shRNA-NC or shRNA-FGF2. (e) FGF2 protein levels were detected by western blot analysis in A549 cells transfected with FGF2 or control shRNA. * $P < 0.05$, ** $P < 0.01$ vs control.

CCK-8 assays (Figure 6a) as well as *in vitro* migration and invasion assays (Figures 6b and c) revealed that overexpression of FGF2 significantly reversed the tumor suppressive effects of

miR-152 on A549 cells. These effects were validated using another NSCLC cell line, H460. Increased levels of FGF2 mRNA after cells were transfected with an FGF2 plasmid were

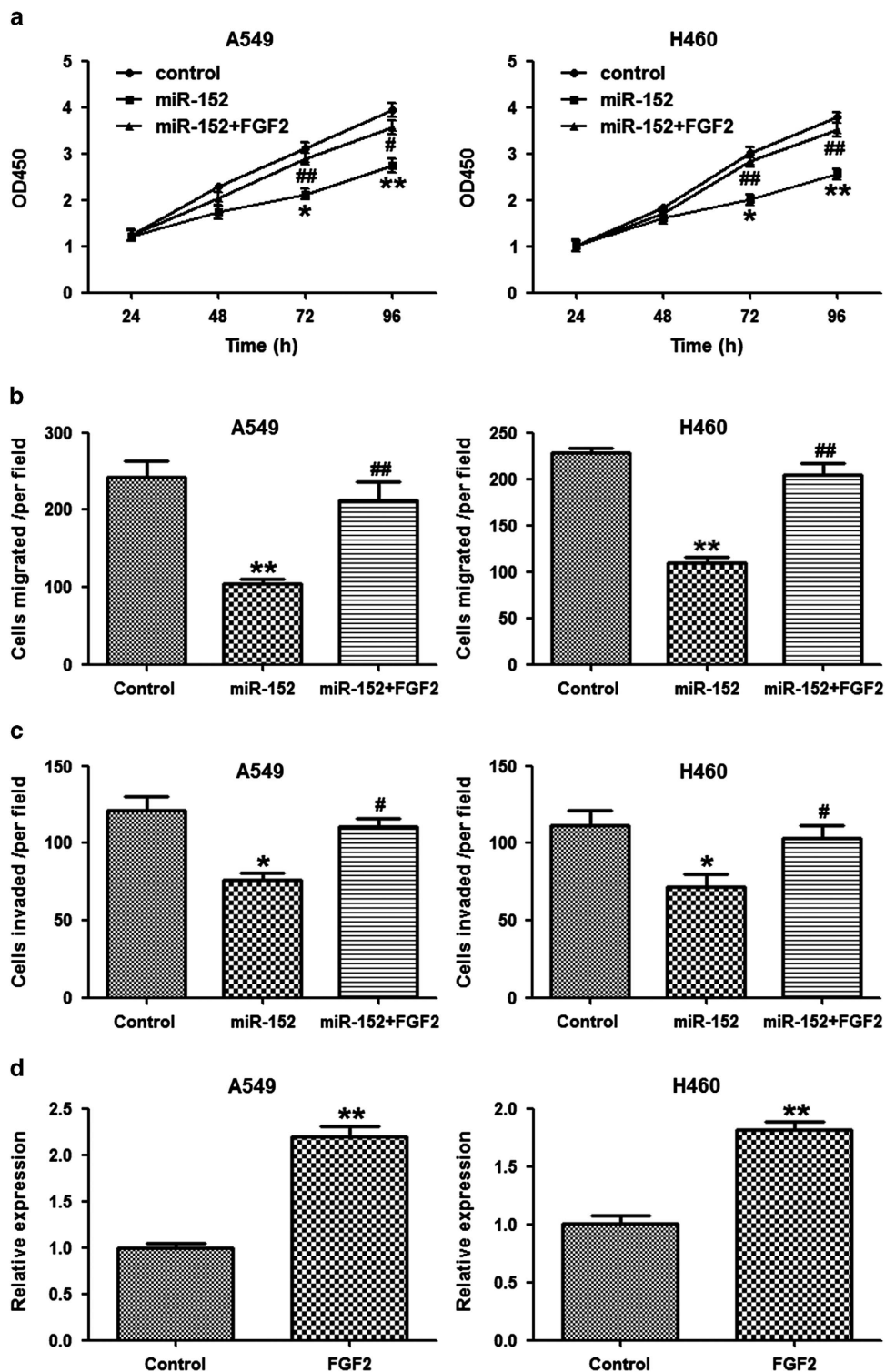


Figure 6 Fibroblast growth factor 2 (FGF2) overexpression partially attenuated the tumor suppressive effect of microRNA-152 (miR-152). (a–c) A549 or H460 cells were transfected with miR-152 or control mimic (control) with or without an FGF2-overexpression plasmid. Proliferation (CCK-8 assay) (a), *in vitro* migration (b) and *in vitro* invasion (c) were evaluated as described in the Materials and methods. (d) FGF2 expression in A549 or H460 cells transfected with an FGF2-overexpression plasmid or the empty vector (control) was measured by quantitative real-time PCR (qRT-PCR). * $P < 0.05$, ** $P < 0.01$ vs control. # $P < 0.05$, ## $P < 0.01$ vs miR-152 group.

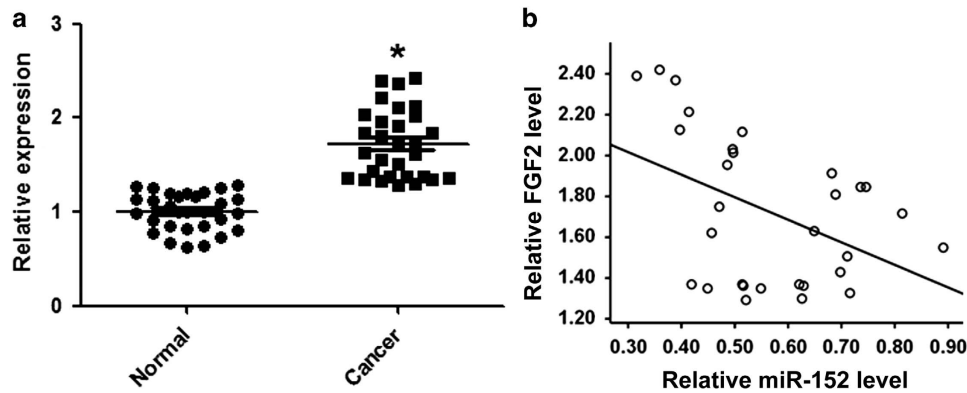


Figure 7 Fibroblast growth factor 2 (FGF2) levels were inversely correlated with microRNA-152 (miR-152) in non-small-cell lung cancer (NSCLC) tissues. (a) FGF2 expression levels in 30 pairs of NSCLC tissues and their matched normal tissues were measured by quantitative real-time PCR (qRT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (b) Correlation analysis between FGF2 mRNA levels and miR-152 expression in NSCLC tissues (Spearman's correlation analysis, $r = -0.422$; $P < 0.05$). * $P < 0.05$ vs control.

confirmed by qRT-PCR (Figure 6d). These data indicate that restoration of FGF2 significantly attenuated the tumor suppressive effect of miR-152.

FGF2 expression was inversely correlated with that of miR-152 in NSCLC tissues

To further explore the relationship between FGF2 and miR-152 *in vivo*, we examined the expression of FGF2 in 30 pairs of NSCLC tissues and their matched normal tissues using qRT-PCR. We found that FGF2 expression was significantly increased in NSCLC tissues relative to the matched controls (Figure 7a). Moreover, FGF2 was negatively correlated with miR-152 expression in the same NSCLC tissues (Figure 7b). These data further indicate that FGF2 was a target of miR-152 in NSCLC.

DISCUSSION

The miRNAs have been reported to play essential roles in carcinogenesis and tumor progression.^{19,20} In addition, acting as either tumor suppressors or oncogenes, miRNAs are involved in several aspects of cancer biology including cell proliferation, apoptosis, migration and invasion.^{21,22} In this study, we focused on miR-152 that belongs to the miR-148/152 family that includes miR-148a, miR-148b and miR-152. Many studies have revealed that members of the miR-148/152 family potentially act as oncogenes and tumor suppressors in different cancers.²³ For example, the expression of miR-148/152 family members was found to be decreased in cholangiocarcinoma, endometrial serous adenocarcinoma, gastrointestinal cancers, hepatocellular carcinoma, oral squamous cell carcinoma, ovarian cancer, pancreatic cancer and prostate cancer, and these family members may function as tumor suppressors in these tumors.²³ Azizi *et al.*²⁴ found in pancreatic cancer cell lines that miR-148b and miR-152 reactivated tumor suppressor genes by inhibiting DNA methyltransferase-1 (DNMT1). Zhu *et al.*¹⁵ showed that overexpression of miR-152 inhibited the migratory and

invasive capabilities of prostate cancer cells *in vitro* and that low miR-152 expression correlated with advanced pathological T stages. Moreover, Xiang *et al.*²⁵ reported that overexpression of miR-152 increased the sensitivity of ovarian cancer cells to cisplatin by inhibiting proliferation and promoting apoptosis through targeting of DNMT1.

However, the role of miR-152 in NSCLC carcinogenesis remains unclear. A recent report by Su *et al.*¹⁸ revealed that miR-152 suppressed the proliferation, colony formation, migration and invasion of NSCLC cells by targeting ADAM17. In this study, we showed that miR-152 was markedly decreased in NSCLC tissues and cell lines. The ectopic overexpression of miR-152 effectively inhibited NSCLC cell proliferation, enhanced apoptosis and suppressed migration and invasion. These results suggest that miR-152 is a novel tumor suppressive miRNA in NSCLC. Many previous studies of miR-152 in various cancers focused on its relationship with DNA methylation.^{26–28} However, DNA methylation may not be the only target of miR-152, as another target molecule, ADAM17, has been recently reported. Indeed, our study suggests another miR-152 target, FGF2, in NSCLC cells.

To elucidate the molecular mechanisms involved in miR-152-induced inhibition of NSCLC growth and invasion, we used TargetScan 6.2 to predict miR-152 target genes. FGF2, which is frequently increased in many malignancies including NSCLC,^{29–31} was identified as a target of miR-152 in NSCLC cells. The inhibition of FGF2 expression resulted in similar effects as the overexpression of miR-152, and the restoration of FGF2 markedly attenuated the tumor suppressive effects of miR-152 on NSCLC cells. Furthermore, FGF2 levels were increased and were negatively correlated with miR-152 levels in NSCLC tissues. The miRNAs can induce mRNA degradation or translational suppression, and downregulated mRNA levels may result in decreased protein levels. Thus, the observed decrease in FGF2 protein levels induced by miR-152

might be because of the degradation of FGF2 mRNA. Together, these data suggest that miR-152 inhibits NSCLC growth and metastasis by inhibiting FGF2. In various cancers, FGF2 acts as an essential regulator of cell proliferation and metastasis.^{32,33} Coleman *et al.*³⁴ found that in pancreatic stellate cells, the nuclear translocation of FGF2 facilitated their invasion. Rades *et al.*³⁵ reported that FGF2 was an independent prognostic factor for patients with locally advanced squamous cell carcinoma of the head and neck. FGF2 also plays essential roles in tumor angiogenesis. For example, Zhou *et al.*³⁶ reported that miR-503 suppressed tumor angiogenesis and growth by targeting FGF2 and vascular endothelial growth factor A. Fons *et al.*³⁷ reported that tumor vasculature could also be regulated by FGF/FGF receptor signaling-mediated angiogenesis. Here, our work further investigated the role of FGF2 in NSCLC.

In conclusion, this study demonstrated that miR-152 was significantly decreased in NSCLC tissues and cell lines. The exogenous overexpression of miR-152 inhibited tumor growth and metastasis of NSCLC cells through targeting of FGF2. Together, our data provide novel insights into therapeutic applications for treating NSCLC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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